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Immunohistochemical Detection of Transforming Growth Factor- β_1 in Fibrotic Liver Diseases

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Transforming growth factor- β_1 was localized by means of immunohistochemical reaction in liver biopsy specimens taken from patients having different chronic liver diseases with extending fibrosis. Two polyclonal antibodies that were produced in rabbits were directed against the amino terminal of transforming growth factor- β_1 .

Staining by anti-CC(1-30) was primarily extracellular and located in the portal and periportal fibrotic areas of all seven cases with chronic active hepatitis. No staining was noted in the four chronic persistent cases studied. A strong reaction was seen with the antibody in nine of the ten cirrhotic samples, whereas it was negative in one inactive cirrhosis case and in all five cases with normal liver histological findings. No positive staining could be detected by the anti-LC(1-30) in any of the liver tissues.

Detection of transforming growth factor- β_1 in active liver diseases at the site of fibrosis suggests that transforming growth factor- β_1 might have a role in the process and progression of fibrosis during the development of the disease. (HEPATOLOGY 1991;14:269-273.)

Increased deposition of extracellular matrix is a characteristic change in several chronic liver diseases, especially in cirrhosis (1-4). The exact factors that are responsible for this accumulation are unknown. Considering the similarities in histopathological features for cirrhosis and chronic hepatitis, as well as the polyetiological origin of both, we considered common factors that could be responsible for fibrosis in the liver during several pathological conditions. One candidate for such a factor is transforming growth factor- β_1 (TGF- β_1).

TGF- β_1 is a multifunctional growth factor. It is presumed to play a role in the regulation of many physiological and pathological processes (5). The first recognized *in vivo* effect was the stimulation of wound healing (6). The deposition of extracellular matrix is the most important event during the process of scar formation in the late phase of wound healing. For this reason, it was proposed that TGF- β_1 may have an influence on the metabolism of extracellular matrix

constituents. In various experimental and *in vivo* processes, TGF- β_1 has been reported to cause an increase in the synthesis of different matrix components (collagen, fibronectin, etc.) (7) while also inhibiting their proteolytic degradation (8, 9). Furthermore, TGF- β_1 has been reported to increase the number of receptors for extracellular matrix components on fibroblasts (10). Because the ability of TGF- β_1 to increase matrix accumulation in various fibrotic processes, TGF- β_1 may also have a direct function in the regulation of progressive liver fibrosis. There are some data supporting this idea in experimental systems (11-14). In this study, we used immunohistological methods to detect TGF- β_1 in human liver biopsy specimens from patients with progressive fibrotic liver diseases.

MATERIALS AND METHODS

In a retrospective study, we analyzed the needle biopsy specimens from 26 patients; 7 with CAH, 4 with chronic persistent hepatitis, and 10 with alcoholic HBsAg-negative cirrhosis. Five liver biopsy specimens in which no pathological alterations were seen were included as controls. The biopsy specimens were fixed in 8% neutral formaldehyde and embedded in paraffin. The diagnosis of liver disease was made from slides stained with hematoxylin and eosin, PAS, Gomori and Mallory's trichrome procedures.

Immunohistochemistry

The antibodies used in this study were produced and characterized for TGF- β , specificity by Ellingsworth et al. (15) and Flanders et al. (16). The polyclonal antisera were generated in rabbits by two different synthetic preparations of a peptide corresponding to the amino terminal 30 amino acids of TGF- β_1 (16, 17). These two antisera give different staining patterns in several tissues and are believed to differentiate between the synthesized and activated forms of TGF- β_1 in some experimental systems. Staining with anti-LC(1-30) is principally intracellular, whereas that of anti-CC(1-30) is extracellular. It was suggested that anti-LC(1-30)-positive cells could be the sites of TGF- β_1 synthesis, whereas anti-CC(1-30) may recognize activated TGF- β_1 (17). A computer search of the immunogen sequence revealed no homology with five or more adjacent amino acids of any protein in the database with the exception of TGF- β_2 (18). Therefore it is unlikely that cross-reactivity with other proteins is responsible for the staining patterns.

After deparaffinization, the blocking of endogenous peroxidase was performed in H_2O_2 /methanol. Membrane perme-

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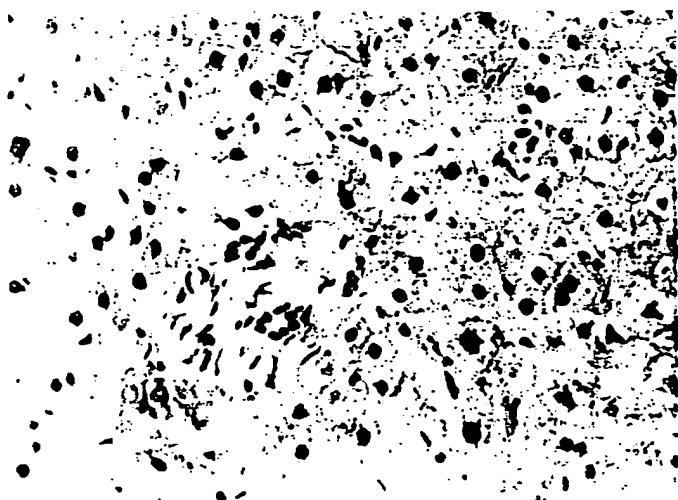


FIG. 1. Normal liver. Negative reaction with anti-CC(1-30) antibody. (H & E; original magnification $\times 140$.)

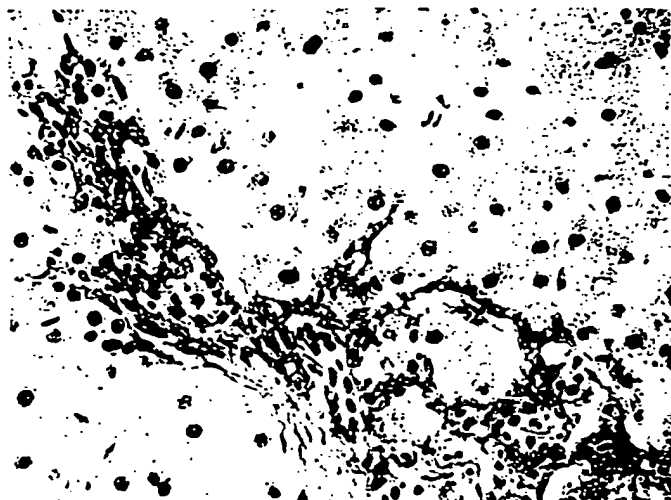


FIG. 3. CAH. Strong immunohistochemical reaction (black staining) in the area of periportal fibrosis. (H & E; original magnification $\times 140$.)



FIG. 2. Chronic persistent hepatitis. Mild lymphocytic infiltration in the portal area. Negative reaction with anti-CC(1-30) antibody. (H & E; original magnification $\times 200$.)

abilization was achieved by treatment of the sections with proteinase K (2 $\mu\text{g}/\text{ml}$ in 20 mmol/L Tris, 2 mmol/L CaCl_2 , pH 7.4) for 15 min at room temperature. Sections were then incubated for 2 hr in humidified chambers at room temperature with the IgG fraction of anti-CC(1-30) or anti-LC(1-30) antiserum (25 $\mu\text{g}/\text{ml}$). Binding was visualized by using a biotin-labeled Biogenex anti-rabbit antibody, with diaminobenzidine as the chromogen. Sections were counterstained with hematoxylin. On control slides, the primary antibody was replaced by normal rabbit IgG.

RESULTS

Anti-LC(1-30) Antibody

Specific binding of anti-LC(1-30) antibody could not be detected on liver sections from healthy or diseased

patients. Usually, we noted a faint yellow staining overall with some darker spots independent of the histological structures and diagnosis.

Anti-CC(1-30) Antibody

Normal Liver. The normal liver findings were essentially negative (Fig. 1). Occasionally, we noted a faint reaction over some sinusoidal cells or yellow stripes above portal triangles, but this staining was very weak and not significant.

Chronic Persistent Hepatitis. In the four cases studied, the result was similar to the normal livers. Positive immunostaining was not observed either on the normal liver structures or on the circumscribed inflammatory lesions (Fig. 2).

CAH. In each of the seven cases of CAH, a distinct positive reaction was seen in the area of portal and periportal inflammation (Figs. 3 and 4). The staining was almost exclusively extracellular, involving the periphery of the liver lobules corresponding to the areas of the piecemeal necrosis. At times, the staining surrounded the trapped hepatocytes. The pattern of immunostaining corresponded closely with the distribution of extracellular matrix material seen on slides stained by Mallory's trichrome method. In two cases, mild intra-lobular inflammation without fibrosis was observed. $\text{TGF-}\beta_1$ staining was not detected in these areas of inflammation.

Cirrhosis. Seven of the cirrhotic cases corresponded to the active florid cirrhosis. Heavy inflammatory reaction consisting mainly of lymphocytes was present in the septa surrounding the pseudolobules. Occasionally, 5 to 10 hepatocytes formed groups that were surrounded by thin connective tissue fibers and infiltrated by inflammatory cells. In these specimens the increased amount of extracellular matrix associated with inflammation was positively stained. The brownish yellow reaction

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FIG. 4. CAH. Strong immunohistochemical reaction (black staining) in the area of portal and periportal fibrosis. (H & E; original magnification $\times 140$.)

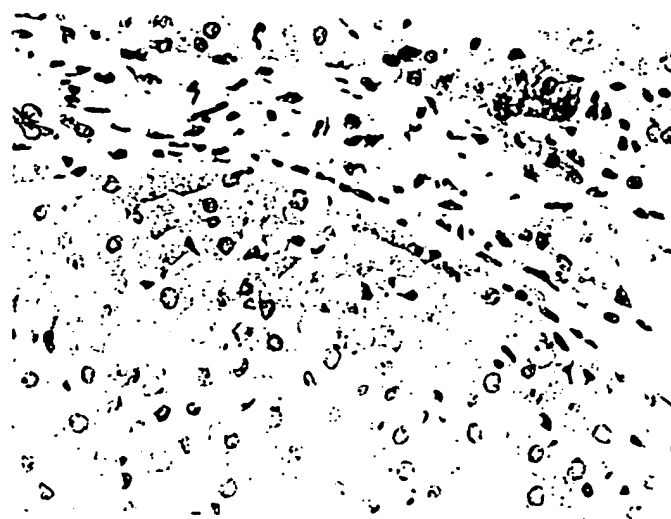


FIG. 6. Inactive cirrhosis. Negative reaction in the septa. (H & E; original magnification $\times 140$.)



FIG. 5. Active cirrhosis. Heavy positive reaction in the connective tissue surrounding the pseudolobule. (H & E; original magnification $\times 140$.)



FIG. 7. Inactive cirrhosis. The thick fibrotic septa are primarily negative. Positive staining can be seen only at the border between the parenchyma and connective tissue. (H & E; original magnification $\times 26$.)

appeared to circumscribe the pseudolobules (Fig. 5) and also spread into the pseudolobules in places of pericellular fibrosis.

Three cirrhotic patients had "burnout" or inactive cirrhosis. The pseudolobules in these cases were circumscribed by thick fibrotic bands with a mild lymphocytic infiltration. In one of these cases, the immunostaining was negative in repeated experiments (Fig. 6). TGF- β_1 could be demonstrated in the two other specimens; however, its distribution was slightly different than in active cirrhosis. The thick fibrotic bands were primarily negative except at the border between the parenchyma and connective tissue (Fig. 7). The inflammatory reaction, and probably the active fibrogenesis, was also localized in this area. Where inflammation and

fine fibrotic septa spread into the pseudolobules, the immunostaining was also positive (Fig. 8c).

In three cirrhotic samples (one inactive and two active), a few endothelial (Fig. 8a) and sinusoidal lining cells (Fig. 8b) also reacted with the anti-CC(1-30) antibody.

DISCUSSION

TGF- β_1 was not detected by immunohistochemistry in normal human liver or in liver from patients with chronic persistent hepatitis. However, in liver biopsy specimens in which an active fibrotic process was observable, namely in chronic aggressive hepatitis and in cirrhosis, we found strong immunostaining for TGF- β_1 . The localization of TGF- β_1 corresponded quite

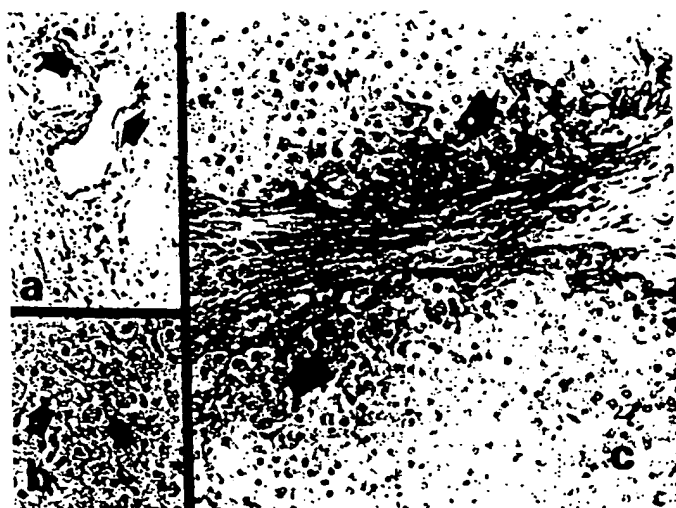


FIG. 8. Inactive cirrhosis. Positive intracellular staining can be seen in some endothelial (a) and sinusoidal lining (b) cells. The fibrotic band is stained only beside the parenchyma where inflammation and fine fibrotic septa spread into the pseudolobule. (H & E; original magnification $\times 70$.)

well to the site of fibrosis or fibrogenesis. This result suggests a connection between TGF- β_1 and fibrogenesis.

It has recently been suggested that TGF- β_1 may influence hepatic fibrogenesis through its effect on the collagen synthesis of fat-storing cells (11, 19). Nakatsukasa et al. (12, 14) described increased expression of TGF- β_1 and collagen type I, III and IV in a CCl₄-induced rat liver fibrosis model. During the early stages of rat liver fibrosis, the increase in expression of the procollagen α_1 (I) gene followed that of TGF- β_1 . Their data suggest that TGF- β_1 , or at least its messenger RNA (mRNA), was produced locally in the fibrotic liver.

In our cases, the immunostaining was mainly extracellular with the anti-CC(1-30) antibody. However, in three cirrhotic cases, we saw positive staining over some sinusoidal and endothelial cells. According to Thompson et al.'s (17) data, the anti-CC(1-30) antibody reacts with the active extracellular form of TGF- β_1 . In our earlier experiments (20), the same antibody reacted positively with the oval cells in rat liver that also contained the TGF- β_1 mRNA according to our *in situ* hybridization data. Thus the positive cells in our recent human samples may be the site of TGF- β_1 production. The value of this finding is limited because we found this cellular staining in only three samples. Considering the data from the literature, it seems reasonable to suggest that TGF- β_1 may be produced by the liver's endothelial and sinusoid lining cells. Nakatsukasa et al. (12, 14), studying the TGF- β_1 expression by *in situ* hybridization in their rat liver fibrosis model, localized the message in the endothelial, desmine-positive perisinusoidal and periductal cells. There are data from other experimental models that localize the TGF- β_1 production in the endothelial and sinusoidal cells (21-23). The anti-LC(1-30) antibody is thought to recognize the intracellular inactive form of TGF- β_1 ; however, similar to

Thompson et al.'s (17) data, we could not see any positive staining with this antibody in the human liver. Czaja et al. (13) reported positive staining in mouse liver with the anti-LC(1-30) antibody in a schistosomiasis-induced liver fibrosis model. It occurred predominantly in hepatocytes and to a lesser extent in endothelial and Kupffer cells. The reason for this difference is at present unknown.

TGF- β_1 expression is increased in the liver after partial hepatectomy (21). Its production can also be induced in the liver by several hepatotoxic chemicals, such as diethylnitrosamine, 2-acetylaminofluorene (20), CCl₄ (12), aflatoxin, tetrachlorodibenzo-*p*-dioxin and phenobarbital (unpublished observations). Hill et al. (24) found an increased level of TGF- β_1 expression in fetal rat liver, and by biological assay, they proved the presence of the protein as well. Strain et al. (25), studying the effect of TGF- β_1 on human fetal liver cells, concluded that it may have influenced hepatocyte differentiation, as had been reported for other cell types. Our earlier *in vitro* and *in vivo* experimental results supported this hypothesis (20). Together these data imply that TGF- β_1 may have a function in liver restoration after injuries induced by chemical, mechanical or other means. However, after repeatedly occurring injuries or with the coexistence of different harmful effects, TGF- β_1 may be produced in large amounts or for extended periods. This may lead to pathological reactions, such as liver fibrosis or cirrhosis. Or, as we found in our rat hepatocarcinogenesis model (20), it may even play the role of endogenous promoter. If an increased amount of TGF- β_1 in the liver is a common factor in cirrhosis caused by viral infections, alcohol consumption or other chemical injuries, and at the same time it has endogenous promoter potential, TGF- β_1 may play a key role in the sequence of events from hepatitis to cirrhosis and in the development of HCC.

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REFERENCES

1. Martinez-Hernandez A. The hepatic extracellular matrix. II. Electron immunohistochemical studies in rats with CCl₄-induced cirrhosis. *Lab Invest* 1985;53:166-186.
2. Galambos JT, Shapira R. Natural history of alcoholic hepatitis. IV. Glycosaminoglycans and collagen in hepatic connective tissue. *J Clin Invest* 1973;52:2952-2962.
3. Murata K, Ochiai Y, Akashio K. Polydispersity of acidic glycosaminoglycan components in human liver and the changes at different stages in liver cirrhosis. *Gastroenterology* 1985;89:1248-1255.
4. Popper H. Pathologic aspects of cirrhosis: a review. *Am J Pathol* 1977;87:228-264.
5. Roberts AB, Sporn MB. The transforming growth factor-betas. In: Sporn MB, Roberts AB, eds. *Handbook of Experimental Pharmacology*. Vol. 95/I. Heidelberg: Springer Verlag, 1990:419-472.
6. Sporn MB, Roberts BA, Shull JH, Smith JM, Ward JM, Sodek J.

- Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo. *Science* 1983;219:1329-1331.
7. Ignatz RA, Masegüe J. Transforming growth factor beta stimulates the expression of fibronectin and collagen and their incorporation into the extra-cellular matrix. *J Biol Chem* 1986;261:4337-4345.
8. Edwards DR, Murphy G, Reynolds JJ, Whitman SE, Docherty AJP, Angel P, Heath JK. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 1987;6:1899-1904.
9. Laiho M, Sakesela O, Andreasen PA, Keski-Oja J. Enhanced production and extracellular deposition of the endothelial type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor beta. *J Cell Biol* 1986;103:2403-2410.
10. Ignatz RA, Masegüe J. Cell adhesion protein receptors as targets for transforming growth factor beta. *Cell* 1987;51:189-197.
11. Weiner FR, Gianbrone MA, Takahashi S, Annoni G, Czaja MJ, Zern MA. Modulators of Ito cell collagen synthesis [Abstract]. *HEPATOLOGY* 1988;8:1253.
12. Nakatsukasa H, Nagy P, Evarts RP, Hsia C-C, Marsden E, Thorgeirsson SS. The cellular distribution of TGF-beta 1 and procollagen I, III and IV transcripts in carbon tetrachloride induced rat liver fibrosis. *J Clin Invest* 1991 (in press).
13. Czaja MJ, Weiner FR, Flanders KC, Gianbrone M-A, Wind R, Biempica L, Zern MA. In vitro and in vivo association of transforming growth factor beta-1 with hepatic fibrosis. *J Cell Biol* 1989;108:2477-2482.
14. Nakatsukasa H, Evarts RP, Hsia C, Thorgeirsson SS. TGF-beta-1 and type I procollagen transcripts during regeneration and early fibrosis of rat liver. *Lab Invest* 1990;63:171-181.
15. Ellingsworth LR, Brennan JE, Fok K, Rosen DM, Bentz H, Piez KA, Seyedin SM. Antibodies to the N terminal portion of cartilage inducing factor A and transforming growth factor beta. *J Biol Chem* 1986;261:12362-12367.
16. Flanders KC, Thompson NL, Cissel DS, Ellingsworth LR, Roberts AB, Sporn MB. Epitope-dependent immunohistochemical localization of transforming growth factor beta 1. *J Cell Biol* 1989;108:653-660.
17. Thompson NL, Flanders KC, Smith JM, Ellingsworth LR, Roberts AB, Sporn MB. Cell type specific expression of transforming growth factor beta 1 in adult and neonatal mouse tissue. *J Cell Biol* 1989;108:661-669.
18. Marquardt H, Lioubin MN, Ikeda T. Complete amino acid sequence of human transforming growth factor type beta 2. *J Biol Chem* 1987;262:12127-12131.
19. Davis BH. Transforming growth factor beta responsiveness modulated by extracellular collagen matrix during hepatic Ito cell culture. *J Cell Physiol* 1988;136:547-553.
20. Nagy P, Evarts RP, McMahon JB, Thorgeirsson SS. Role of TGF-beta 1 in normal differentiation and oncogenesis in rat liver. *Molec Carcinog* 1989;2:345-354.
21. Braun L, Mead JE, Panzica M, Mikumo R, Bell GT, Fausto N. Transforming growth factor beta mRNA increases during liver regeneration: a possible paracrine mechanism of growth regulation. *Proc Natl Acad Sci USA* 1988;85:1539-1543.
22. Braun L, Gruppuso P, Mikumo R, Fausto N. Transforming growth factor beta 1 in liver carcinogenesis: messenger RNA expression and growth effects. *Cell Growth & Differentiation* 1990;1:103-111.
23. Evarts RP, Nakatsukasa H, Marsden ER, Hsia C-C, Dunsford HA, Thorgeirsson SS. Cellular and molecular changes in the early stages of chemical hepatocarcinogenesis. *Cancer Res* 1990;50:3439-3444.
24. Hill DJ, Strain AJ, Milner RDG. Presence of transforming growth factor beta like activity in multiple fetal rat tissues. *Cell Biol Int Rep* 1986;10:915-922.
25. Strain AJ, Hill DJ, Milner RDG. Divergent action of transforming growth factor beta on DNA synthesis in human foetal liver cells. *Cell Biol Int Rep* 1986;10:855-860.